

A SOLUBLE INTESTINAL DIPEPTIDASE FROM PIG
Purification and characterization

by
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This dissertation is based on the following publications:

- I. O. Norén, H. Sjöström and L. Josefsson (1973)
Studies on a soluble dipeptidase from pig intestinal
mucosa. I. Purification and specificity.
Biochim.Biophys.Acta 327, 446-456.

- II. O. Norén
Studies on a soluble dipeptidase from pig intestinal
mucosa. Enzymatic properties.
Acta Chem.Scand. Submitted for publication.

- III. O. Norén and H. Sjöström
Studies on a soluble dipeptidase from pig intestinal
mucosa. Structural properties.
Acta Chem.Scand. Submitted for publication.

The publications are referred to by their Roman numerals.
This dissertation also contains a few unpublished results
labelled (unpublished) in the text. Unless otherwise stated
all amino acids mentioned in the text are L-forms.

English was revised by W.F. Salisbury.

INTRODUCTION

DEVELOPMENT OF THE DIPEPTIDASE RESEARCH

Early studies and classification.

At the end of the nineteenth century, a controversy arose concerning the final digestion and absorption of the dietary protein in the intestine. Cohnheim (1901) showed that an extract of a dog intestine was able to transform a pepton solution (a muscle extract treated with pepsin) into crystalline substances. He concluded that the process responsible for the transformation was of enzymatic nature, and the enzyme was called erepsin. Further studies showed that erepsin was composed of several enzymes. A polypeptidase activity was shown to be different from a dipeptidase and prolinase activity in extracts from both bakers yeast and intestine. Furthermore, a leucyl-peptidase was distinguished from the dipeptidase activity in the small intestine of the pig (for refs., see Johnson and Berger 1942, Smith 1951a,b).

The possibilities of studying the specificity of proteolytic enzymes were considerably improved, when Bergmann and Zervas (1932) developed a method to synthesize optically active peptides composed of almost any amino acids. By systematically varying the side chains and the end groups of the peptides used as substrates, Bergmann (1942) was able to extend the classification scheme of the Willstätter school which emphasized that the chain length of the peptide was responsible for the specificity with the proteolytic enzymes (Grassmann and Schneider 1936). The exopeptidases (Bergmann and Fruton 1937) are usually further divided into the aminopeptidases, carboxypeptidases, and the dipeptidases. To these three groups, the pyrrolidonecarboxyl peptidease (Armentrout and Doolittle 1969) can be added. The

arylamidases, enzymes hydrolyzing amino acyl- β -naphthylamides, are probably aminopeptidases, as purified arylamidase preparations showed aminopeptidase activity (Little and Behal 1971, Maroux et al. 1973).

The definition of a dipeptidase was early stated (Grassmann et al. 1935, Bergmann et al. 1935): A dipeptidase should only hydrolyze dipeptides having free α -carboxyl- and α -amino-groups; furthermore, the hydrogen of the peptide bond and the L-form of the amino acids were necessary. It is suggested, however, that the two latter criteria should be excluded from the definition of a dipeptidase, as two enzymes purified to homogeneity did not fulfil them, despite having exclusively dipeptides as substrates (Campbell et al. 1966, Sjöström et al. 1973).

Johnson and Berger (1942) in their review concluded that erepsin, earlier believed to be one enzyme, was composed of aminopeptidases and a plurality of dipeptidases, which concerning the dipeptidases was a new view as it was earlier believed that one single dipeptidase was responsible for all the dipeptidase activity. The work of Smith and his colleagues during the 1940s and 1950s verified the existence of several different dipeptidases, resulting in a further classification of this group of enzymes:

- Glycyl-glycine dipeptidase (Smith 1948b)
- Glycyl-L-leucine dipeptidase (Smith 1948c)
- Iminodipeptidase (prolinase), (Davis and Smith 1953)
- Imidodipeptidase (prolidase), (Bergmann and Fruton 1937)
- Carnosinase (Hanson and Smith 1949)
- Cysteinylglycinase (Semenza 1957)

This classification is based on experiments that take advantage of changes in ratios of hydrolysis rates for different substrates during fractionation procedures and inactivation-activation studies.

S t u d i e s o n e n z y m e m e c h a n i s m .

Early, the dipeptidases played a role in the studies aimed at unmasking the details of enzyme mechanism. It was proposed that the substrate glycyl-glycine was attached to the enzyme at two points (diaffinity theory) by the observation that benzylation of the dipeptide's amino group prevented hydrolysis of the dipeptide by intestinal extracts (von Euler and Josephson 1926, Josephson and von Euler 1927). On the basis of the found stereospecificity of the dipeptidases, the diaffinity theory was extended to the polyaffinity theory (Bergmann et al. 1935) which states that the enzyme-substrate complex is connected at three or more points.

Many of the enzymes in the group of exopeptidases were shown to depend on the presence of a metal for their action (Smith 1951a,b). On this basis, Smith et al. (1954) formulated a theory on the catalytic action of metal dependant peptidases - among these, the prolidase and leucine aminopeptidase - by ascribing a central role to the metal in the catalytic action of the enzymes. The selection of enzymes in the subsequent investigations, performed to prove the theory, was partly guided by the possibilities of obtaining homogeneous enzyme preparations. The dipeptidases were found unstable and difficult to purify; they were therefore abandoned in favour of other peptidases. Later, however, Campbell et al. (1967) revived the enzyme mechanistic studies on the renal dipeptidase.

T h e p h y s i o l o g i c a l a n d m e d i c a l i m p o r t a n c e .

So far, the dipeptidase studies have primarily concerned the dipeptidases as molecules, although a great mapping work was done on their distribution among living material (Johnson and Berger 1942). The dipeptidases were later studied in relation to their proposed digestive function and suggested importance in the pathogenesis of coeliac disease (for refs., see Gray and Cooper 1971, Booth and Dowling 1970).

Intracellular protein metabolism. Protein synthesis has been extensively studied on the molecular level and much information has appeared. On the other hand, little is known about intracellular protein hydrolysis and the enzymes responsible (Lehninger 1970). The cathepsins (Mycek 1970), proteolytic enzymes of lysosomal origin, have been made responsible for the hydrolysis of intracellular proteins. It was found in studies on rat liver (Coffey and de Duve 1968) that lysosomes were capable of hydrolyzing acid denaturated globin into mainly dipeptides, which for further hydrolysis needed the peptidases of the cytosol. These results suggest that the dipeptidases are of importance in the intracellular catabolism of proteins, a suggestion further supported by their wide distribution in different mammalian tissues (Fruton 1946, Smith 1948a, Josefsson et al. 1968a).

Protein digestion and absorption. The gastric and pancreatic proteolytic enzymes are well studied both from biochemical (Boyer 1971) and physiological (Code 1967) standpoint. The information available for the intestinal enzymes in general is quite different. Intestinal peptidases have not until recently been reported purified (Maroux et al. 1971, Sjöström et al. 1971, Norén et al. 1971, Das and Radhakrishnan 1972, Maroux et al. 1973); therefore the present biochemical and physiological knowledge of them is scanty, but it is important for the understanding of peptide absorption and digestion. The physiological function of the dipeptidases has been discussed in relation to their ubiquitous distribution among the organs of mammals (Maschmann 1941, Smith and Bergmann 1944, Fruton 1946, Smith 1948a,b,c, Josefsson et al. 1968a). In the discussion, it was stated that the dipeptidases of the intestinal mucosa do not necessarily have a digestive function (Smith 1948a); on the other hand, its richness in dipeptidase activity could reflect an adaptation to a digestive function (Josefsson et al. 1968a). Rhodes et al. (1967) proposed that the aminopeptidases found in the brush border membrane of the enterocyte might be responsible

for the digestive peptidase function of the small intestine. The distribution of the dipeptidases and aminopeptidases between cytosol and brush border membrane has been reported to be quite different - about 90 % of the dipeptidase activity of the cell is found in the cytosol, whereas the distribution of the aminopeptidase activity is more in favour of the brush border membrane (Robinson 1963, Josefsson and Sjöström 1966, Rhodes et al. 1967, Peters 1970a, Fujita et al. 1972, Kim et al. 1972, Maroux et al. 1973). The biological significance of this distribution pattern is uncertain, but it is tempting to ascribe a digestive role to the membrane bound enzymes, although the difference in distribution perhaps only reflects differences in their binding mechanisms and physicochemical properties. A common model describing the final digestion and absorption of oligopeptides in the small intestine is that in which the oligopeptides are hydrolyzed to amino acids and dipeptides by the brush border bound peptidases and in which the further hydrolysis of the dipeptides is performed by an intracellular "dipeptidase sieve" (Peters 1969, 1970b, Booth 1970).

For a long time, the predominant opinion was that proteins were degraded to amino acids before they were absorbed by the small intestine (Wiseman 1968). But the experiments performed by Newey and Smyth (1960) questioned this simple view. These investigators showed that a dipeptide could be absorbed as such before the intracellular hydrolysis. Investigations performed by Ugolev et al. (1964) pointed to a peptide hydrolysis localized to the brush border membrane, a finding which does not conflict with the experiments of Newey and Smyth (Smyth 1972). Quite recently, results have been reported supporting the view that the uptake of dipeptides from the intestinal juice is of quantitative importance under physiological conditions (Craft et al. 1968, Matthews et al. 1968, Adibi and Morse 1971, Hellier et al. 1972a). Support for the existence of separate dipeptide transporting mechanism(s) different from those of the amino acids has been gained by the demonstration that patients suffering from inborn errors of amino acid absorption (Cystinuria and Hartnups disease)

have undisturbed absorption of dipeptides in their small intestines (Hellier et al. 1972b, Asatoor et al. 1972, Milne 1972). These findings have tempted the reseachers in the field to propose an absorption model for peptides in which the transport and hydrolysis mechanisms are spatially, closely related to each other (Matthews 1972, Ugolev 1972). The dipeptidases of the small intestine thus might function in a close relation to the entry mechanism of the dipeptides.

The pathogenesis of coeliac disease. Gliadin, a protein fraction of flour, has been blamed for the toxic effect on the intestines of persons suffering from the coeliac disease (Van de Kamer et al. 1953). Experimental results suggest that a peptidase deficiency might be an underlying factor in the pathogenesis of coeliac disease (for refs. see Dahlqvist et al. 1970). Dipeptidase activity in the intestinal mucosa of such patients was below the normal values (Dahlqvist et al. 1970, Berg et al. 1970), but this finding was thought to be a secondary phenomenon, as it was paralleled by a low disaccharidase activity in the damaged intestinal mucosa. Furthermore, when the flattened intestinal mucosa became normal during treatment, the dipeptidase activity increased together with other enzyme activities, indicating that no dipeptidase deficiency had preceded the development of the disease. With the zymogram technique (Lewis and Harris 1967) Dolly et al. (1971) studied the dipeptidase patterns of the intestinal mucosa from normal and coeliac individuals without finding any differences between the two groups. But it must be noted, that the knowledge of the number of peptidases of the intestinal mucosa and their specificity is scanty, making the selection of peptides, serving as substrates, difficult and thereby obscuring the results.

THE GLYCYL-L-LEUCINE HYDROLYTIC ACTIVITY

PREVIOUS INVESTIGATIONS

It has been suggested that the enzyme mainly responsible of the hydrolysis of glycyl-leucine (EC 3.4.3.2) differs from leucine aminopeptidase, prolidase, and glycyl-glycine dipeptidase when studied in preparations of pig intestine, human uterus and rat muscle (Smith 1948c). It was also classified as a dipeptidase.

O c c u r r e n c e .

The glycyl-leucine hydrolytic activity is widely distributed in mammalian tissues and has been demonstrated both in viscera and supporting tissue: Liver, kidney (Maschmann 1941), small intestinal mucosa (Maschmann 1941, Smith and Bergmann 1944), lung, skin, serum (Fruton 1946), heart-, uterine-, and skeletal-muscle (Smith 1948a,c), dental pulp (Schwabe 1969), red blood cells (Lewis and Harris 1967), and trachea and uterine tube epithelium (Josefsson et al. 1968a).

N u m b e r o f h y d r o l y z i n g p r i n c i p l e s .

Studies using the zymogram technique (Lewis and Harris 1967) have shown that up to four electrophoretically separated fractions are able to hydrolyze glycyl-leucine (Dolly and Fottrell 1968, Dolly et al. 1971, Donlon et Fottrell 1972, Fottrell et al. 1972, Kim et al. 1972), but the results of these studies are variable. Studies of the purified exopeptidases showed that pig kidney leucine aminopeptidase (Smith and Spackman 1955) was able to hydrolyze this peptide as was the human liver arylamidase (Little and Behal 1971) and the pig renal dipeptidase (Campbell et al. 1966).

T h e i n t e s t i n a l a c t i v i t y .

Glycyl-leucine hydrolyzing activity has been demonstrated in

the small intestinal mucosa of many different species, and the best conditions for hydrolysis have been determined (Smith and Bergmann 1944, Smith 1948c, Robinson and Shaw 1960, Josefsson and Lindberg 1965a, Josefsson and Lindberg 1966, Lindberg 1966a, Symons and Jones 1966).

Distribution along the gastrointestinal tract. It is well known that different nutrients are preferably absorbed by special parts of the small intestine (cf. the iron and glucose absorption in duodenum and the vitamin B₁₂ absorption in the terminal ileum). With these circumstances in mind, the longitudinal distribution of the glycine-leucine hydrolyzing activity was investigated on rat (Robinson and Shaw 1960, Josefsson and Lindberg 1966), pig (Josefsson and Lindberg 1965b), man (Lindberg 1966a), and sheep (Symons and Jones 1966). The proximal part of the ileum displayed the highest activity, being 3-6 times higher than the activity in the duodenum and terminal ileum. Considerable activity, however, was found in all parts of the small intestine. The velocity of glycyl-leucine absorption also reached a maximum in the proximal part of the ileum (Adibi and Morse 1971).

Distribution in different layers of the intestine. In intestinal juice of different species, low glycyl-leucine hydrolyzing specific activity was found compared to the specific activity of the intestinal mucosa (Symons and Jones 1966, Josefsson et al. 1968b, Adibi and Morse 1971), but in one case, considerable activity was reported (Szafran et al. 1962). This high activity can be explained by the technique used for the collection of intestinal juice, i.e. mechanical irritation (Florey et al. 1941). Using the cryostat technique and cutting the intestine longitudinally, Nordström et al. (1968) reported the highest activity in the apical and middle part of the rat intestinal villi, whereas no activity was found in the crypts and the muscular layer.

Subcellular distribution. The subcellular distribution of the glycyl-leucine hydrolyzing activity has been studied in

different species, about 90 % of the activity of the cell being recovered in the cytosol (Josefsson and Sjöström 1966, Peters 1970a, Kim et al. 1972, Fujita et al. 1972).

Purification. The principles of a purification procedure of a glycyl-leucine hydrolyzing enzyme from pig intestine were described by Norén et al. (1971); later, a purification of a monkey intestinal glycyl-leucine dipeptidase was reported with some data on its specificity and enzymatic properties (Das and Radhakrishnan 1972).

PRESENT INVESTIGATION

This investigation was aimed at purifying in preparative amounts, the intestinal enzyme mainly responsible for the hydrolysis of glycyl-leucine, and at studying its specificity, enzymatic properties, and structural characteristics.

P u r i f i c a t i o n a n d s p e c i f i c i t y (I) .

Source of enzyme. The choice of species to be used as enzyme source was primarily a matter of quantity. The pig was selected for this reason and also because the glycyl-leucine hydrolyzing activity of pig intestinal mucosa has pH and metal dependence similar to the corresponding human activity (Lindberg 1966b). The proximal 2 m intestine was discarded because the glycyl-leucine hydrolyzing activity of this region was found less stable than the corresponding activity of other parts of the intestine, a finding which might be explained by the presence of proteolytic enzymes of the pancreas. The next 3 m intestine was cut into pieces and extracted in water, yielding a quantitative release of the glycyl-leucine hydrolyzing activity (Josefsson and Sjöström 1966). The extract obtained was then lyophilized without great losses of activity, the resulting specific activity of the lyophilized powder being higher than that of a corresponding homogenate owing to the rapid release of the dipeptidase activity in relation to many of the other intestinal proteins. This preparation technique is a convenient way of collecting start material for the further purification in large quantities. The glycyl-leucine dipeptidase activity is stable in the lyophilized intestinal extract when stored at -20°C .

Assay methods. For drawing up the purification procedures, the dipeptidase assay method of Josefsson and Lindberg (1965a) was used, being economical both in dipeptide and enzyme consumption and rapid and simple in daily routine. The rate of hydrolysis of glycyl-leucine was measured at pH 7.8 without the

presence of metals (Josefsson and Lindberg 1965a), but the reaction temperature was lowered from 40 °C to 25 °C for stability reasons.

In the studies of the dipeptide hydrolyzing capacity of the lyophilized intestinal extract, it was necessary for some dipeptides, to use high protein concentrations in the incubation mixtures for sensitivity reasons. As it was difficult to precipitate the protein quantitatively in these concentrations, metaphosphoric acid (2.5 % w/v) replaced ethanol as precipitating agent in the specificity studies.

When peptides containing aromatic side chains were studied, their strong absorbance in the 220 nm region made it necessary to substitute the assay method of Josefsson and Lindberg (1965a) for another assay method operating at a higher wavelength. A method based on 2:4:6-trinitrobenzene-sulphonic acid (TNBS) as an amino group reagent (Binkley et al. 1968) was used with some modifications. Stable absorbance of the solutions after the reaction with the TNBS-reagent could not be achieved; therefore the further development of colour was inhibited by lowering the pH of the reaction solution (Satake et al. 1960) by the addition of trichloroacetic acid.

Stability of the enzyme. Our first attempts to purify the glycyl-leucine hydrolyzing enzyme were hampered by the lability of the enzyme. Glycerol used as stabilizer in early dipeptidase preparations (von Euler and Josephson 1926) appeared to be efficient (FIG. 1) even in moderate concentrations (12.5 % w/v). A further instability emerged as the purification process continued, but it could be managed by the addition of 4 mM 2-mercaptoethanol (FIG. 2). In the last purification step, i.e. the chromatography on Sephadex G-100, the presence of glycerol in the buffer caused disturbances in the chromatography by broadening the peaks. Glycerol, however, could be omitted during the chromatography without great losses of activity, but it was added again immediately after the collection of fractions to maintain the stability of the dipeptidase activity.

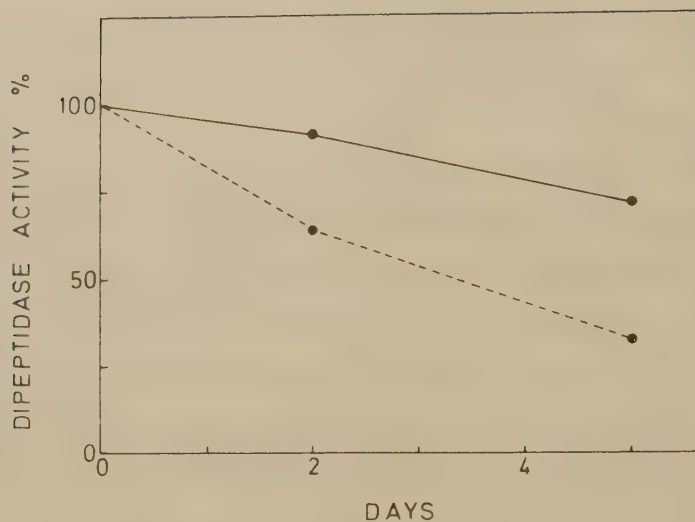


FIG. 1. Stability of the glycyL-leucine dipeptidase activity in 0.05 M sodium phosphate buffer (pH 7.0). Start concentration 10 units of activity per ml. Specific activity 5 units of activity per mg protein. ----- No glycerol added. ——— Glycerol (12.5 %, w/v) added in the buffer.

It was also observed that the glycyL-leucine hydrolyzing activity was inactivated when incubated together with dialysis tubes. When the tubes were pretreated with 0.1 M EDTA at 50 °C overnight, this inactivating action was overcome.

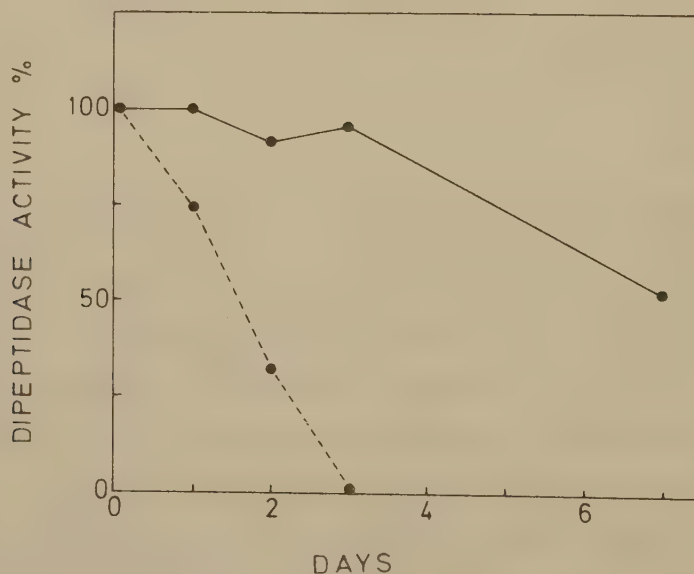


FIG. 2. Stability of the glycyL-leucine dipeptidase activity in 0.05 M Tris-HCl buffer (pH 7.0) containing 12.5 % (w/v) glycerol. Start concentration 13 units of activity per ml. Specific activity 75 units of activity per mg protein. ----- No 2-mercaptoethanol added. ——— 2-mercaptoethanol (4 mM) added.

Purification procedure. The purification of the glycyl-leucine hydrolyzing enzyme was intended to be accomplished without the use of organic solvents, as it has been suggested that these solvents cause changes in the specificity of an aminopeptidase (Nachlas et al. 1962). TABLE I shows the results of a purification experiment.

TABLE I

PURIFICATION OF A DIPEPTIDASE FROM PIG INTESTINAL MUCOSA

Figures are given for 67 g lyophilized intestinal extract. Dipeptidase activity is measured against glycyl-leucine.

Fraction	Total activity (units)	Recovery (%)	Specific activity (units/mg protein)	Purification factor
Supernatant	276 000	100	4.01	1
(NH ₄) ₂ SO ₄ fraction	143 000	52	23.4	5.8
DEAE-cellulose fraction	84 200	31	197	49
DEAE-Sephadex fraction	66 800	24	900	224
Hydroxylapatite fraction	35 500	13	1 320	330
Purified dipeptidase	17 900	6.5	1 380	340

Fractionation with ammonium sulphate was chosen as the first step in the purification procedure. Before preparative fractionations were performed, the solubility of the glycyl-leucine hydrolyzing activity at different ammonium sulphate concentrations was studied. Analytical experiments were carried out with small increments in the ammonium sulphate concentration in a series of solutions of the lyophilized intestinal extract; after centrifugation, the series of supernatants were analyzed for glycyl-leucine hydrolyzing activity. The result of the study gave no basis for a presumption of several enzymes responsible for the activity.

DEAE-cellulose, a resin with good mechanical properties, was well suited for chromatographing the slightly opalescent ammonium sulphate fraction without retardation in flow rate.

The column of DEAE-cellulose was eluted with an increasing linear NaCl-gradient, and the dipeptidase activity appeared as a single peak. Isoelectric focusing (FIG. 3) showed the DEAE-cellulose fraction to be composed of, above all, a dominant protein peak with pI different from that of the glycyl-leucine hydrolyzing activity (pI 5.1, unpublished). This result suggested that further purification of the enzyme could be possible by another ion-exchange chromatography.

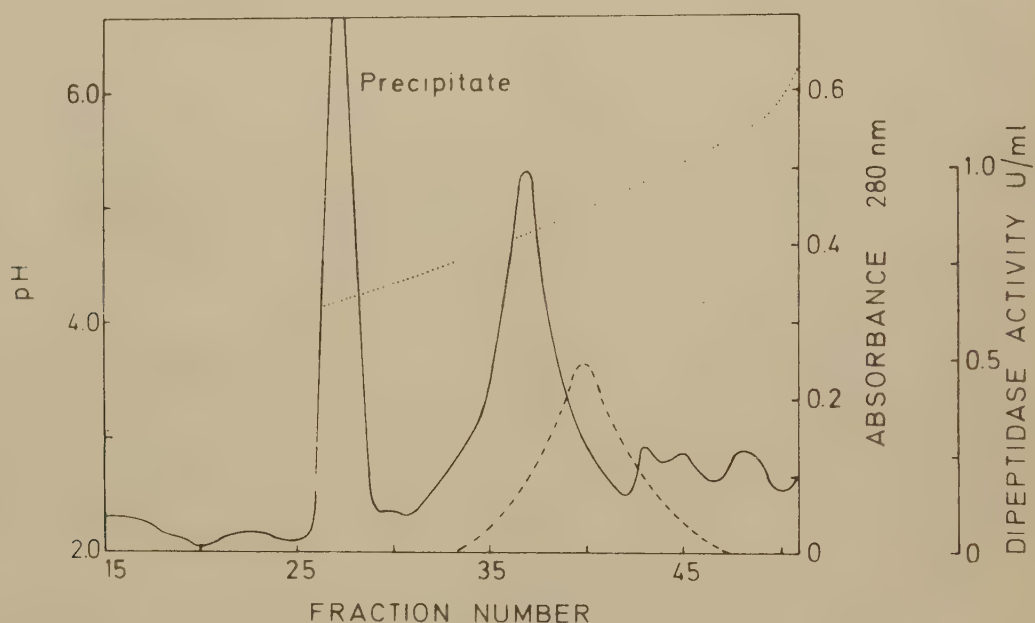


FIG. 3. Isoelectric focusing of the DEAE-cellulose fraction (see TABLE I). 400 units of activity was applied to a 110 ml glass column (LKB-produkter AB, Bromma, Sweden) filled with an Ampholine solution (1 %) and stabilized with a glycerol gradient (0-30 % w/v). 500 V for 48 h was applied. — Absorbance. ----- Dipeptidase activity.pH gradient.

Column chromatography on DEAE-Sephadex A-50, using an increasing linear NaCl-gradient, proved succesful. The glycyl-leucine hydro-lyzing activity appeared well separated from the dominant inactive protein peak; there was also an indication of a connection between the dipeptidase activity and a protein peak. The protein composi-tion of the DEAE-Sephadex fraction was analyzed by polyacrylamide

gel electrophoresis (Davis 1964); the result showed that the DEAE-Sephadex fraction contained two dominant protein components together with about ten other less dominant proteins. One of the dominant components was the glycyl-leucine hydrolyzing enzyme, and it was possible to separate it from the other dominant protein on a column of hydroxylapatite using a stepwise elution with phosphate buffers.

No size-discriminating separation procedure was used earlier in the purification of the enzyme; thus the choice of the gel-filtration technique as the next purification step was natural. The hydroxylapatite fraction was, after concentration by ultrafiltration, chromatographed on a Sephadex G-100 column, and the glycyl-leucine hydrolyzing activity was eluted in two separated peaks, one containing only a small fraction of the total dipeptidase activity coming out in front of a quantitatively dominant peak of smaller molecular size. The dominant dipeptidase peak neatly followed the protein curve in the same part of the chromatogram. This peak was selected as the purified dipeptidase (soluble dipeptidase).

Purity of the enzyme. The purity of the soluble dipeptidase was tested by polyacrylamide gel electrophoresis (Davis 1964) and was found to be 99 % pure. But besides the major band corresponding to the dipeptidase activity, two other faint bands with lower mobility were always present. They occurred even in experiments where the major band, after being eluted, was re-electrophorized. No experiments have been undertaken to study the nature of these faint bands, but they might represent polymeric forms of the soluble dipeptidase (see Immunochemical studies and the pig intestinal prolidase, Sjöström et al. 1973).

The soluble dipeptidase was also subjected to crossed immunoelectrophoresis (Clarke and Freeman 1968) with a polyvalent antibody solution (see Immunochemical studies). Only one precipitate was visible, giving further support to the purity of the preparation.

During the studies on the structural properties of the soluble dipeptidase (III), additional indications on the

purity of the purified dipeptidase were obtained; namely, a homogeneous performance in the gelfiltration and polyacrylamide gel electrophoresis in dodecylsulphate (Fish et al. 1970, Weber et al. 1972) and only one N-terminal amino acid was found by the method of Gray (1972).

Immunochemical studies. A polyvalent rabbit antibody solution was prepared with the DEAE-Sephadex fraction as antigen by the method of Axelsen (1971).

The structural relation between the two dipeptidase peaks obtained in the Sephadex G-100 chromatography was studied with immunochemical technique. The fractions corresponding to the two separated dipeptidase peaks were pooled, giving two different dipeptidase solutions. The solution corresponding to the first eluted dipeptidase peak was concentrated by ultrafiltration 150 times and then together with the soluble dipeptidase (the second eluted dipeptidase peak) run in a fused rocket immunoelectrophoresis (Svendsen and Rose 1970). Two rockets joined by an unbroken precipitate were obtained (FIG. 4, unpublished) indicating immunological identity between the two dipeptidase peaks (Krøll 1968, Grubb 1972, Bock and Axelsen 1972). It was also shown that the first eluted dipeptidase peak was not pure. Thus the two separated dipeptidase fractions are structurally very similar but of different molecular size, suggesting that the first eluted fraction represent a polymeric form of the second.

In order to study the structural relation between the soluble dipeptidase and a glycyl-leucine dipeptidase obtained from another organ of the pig, an immunochemical comparison between the soluble dipeptidase and an isolated brain glycyl-leucine dipeptidase was undertaken.

The brain glycyl-leucine dipeptidase was isolated by extracting pieces of pig brain in water and then lyophilizing the extract solution. The lyophilized brain extract, after dissolution and dialysis, was applied to a DEAE-cellulose column, which was eluted with an increasing linear NaCl-gradient. The glycyl-leucine hydrolyzing activity appeared in the chromatogram as a single peak at the same NaCl concentration as the soluble dipepti-

dase in the same chromatographic system (unpublished).



FIG. 4. Fused rocket immuno-electrophoresis of pooled fractions from a Sephadex G-100 chromatography (I). In the left well 1 μ l of the pooled fractions (0.38-0.43 elution volume/bed volume) was applied. In the right well 1 μ l of pooled fractions (0.32-0.33 elution volume/bed volume) was applied after being concentrated about 150 times. After application the plate was allowed to stand for diffusion 2 h. The electrophoresis was then performed applying 3 V/cm for 20 h.

The glycyl-leucine hydrolyzing activity appeared in the chromatogram as a single peak at the same NaCl concentration as the soluble dipeptidase in the same chromatographic system (unpublished). The fractions containing the glycyl-leucine hydrolyzing activity were pooled and concentrated by ultrafiltration to 250 units of dipeptidase activity per ml. This concentrate and the soluble dipeptidase were then immunologically compared in the fused rocket immunoelectrophoresis (Svendsen and Rose 1970), and two rockets of the same density, joined by an unbroken line, were obtained (FIG 5, unpublished). The result indicates, that the soluble dipeptidase was structurally similar to a protein, probably a glycyl-leucine dipeptidase, in the brain; this finding strengthens the conclusion that the intestinal dipeptidase are of the same nature as those obtained from other tissues (Josefsson et al. 1968a).

FIG. 5. Fused rocket immunoelectrophoresis of the soluble intestinal dipeptidase and the isolated brain dipeptidase (I). In the left well 2 μ l (2 units of activity) of the intestinal soluble dipeptidase was applied and in the right well 4 μ l (1 unit of activity) of the concentrated brain preparation was applied. The plate was left for diffusion for 2 h. The electrophoresis was then performed applying 3 V/cm for 20 h.



Specificity studies. The peptides used in this study were tested for their hydrolysis, with both the lyophilized intestinal extract and the soluble dipeptidase as enzymes. The results are presented in TABLES II, III, and IV. Leucineamide was also included in the study (unpublished).

These studies were aimed at getting information on the specificity of the soluble dipeptidase and also of its relation to other peptidase activities present in the lyophilized mucosal extract. The selection of substrates was primarily undertaken to achieve as wide a chemical variation of the peptides as possible. Certain peptides were also included in the study to compare the specificity of the soluble dipeptidase with other distinct exopeptidases. In order to settle the detailed chemical composition of a peptide to be hydrolyzed by the soluble dipeptidase the changes in the rate of hydrolysis were studied by using dipeptides substituted in or lacking end groups, being stereoisomers or containing a β -amino acid (TABLE IV). No trials were made to optimize the reaction conditions in respect of pH, metal content,

and substrate concentration for the different peptides studied, but a standard pH (7.4) and standard substrate concentration (8.5 mM, for alanyl-proline 1.8 mM) were selected.

TABLE II

RELATIVE RATES OF HYDROLYSIS FOR DIFFERENT SUBSTRATES OF THE
INTESTINAL MUCOSA EXTRACT AND THE PURIFIED DIPEPTIDASE

The enzyme activities are expressed as relative rate of hydrolysis of the various substrates in percentage of the hydrolysis rate of glycyl-leucine. Zero per cent activity means that the activity found was less than 0.1 per cent of that against glycyl-leucine. ++ means high activity.

Substrate	Assay method	Hydrolysis (%)	
		Extract	Dipeptidase
Gly-Leu	A	100	100
Ala-Ala	A	280	300
Ala-Leu	A	39	53
Leu-Ala	A	27	35
Ala-Ser	A	190	170
Ser-Ala	A	60	52
Ala-Glu	A	59	56
Glu-Ala	A	11	7.3
Ala-Lys	A	20	19
Lys-Ala	A	3.7	0
Ala-Pro	A	5.4	0
Pro-Ala	A	3.7	2.0
Ala-Tyr	B	9.3	7.3
Tyr-Ala	B	27	14
Ala-Trp	B	77	37
Trp-Ala	B	6.2	0
Gly-Gln	A	38	18
Gln-Gly	A	30	28
Gly-Gly	B	3.1	0.25
Leu-Tyr	B	16	12
Leu-NH ₂	A	15	2.2
Ala-Ala-Ala	A	++	0
Gly-Leu-Tyr	B	++	0

A, Spectrophotometric method (Josefsson and Lindberg 1965 a).

B, TNBS-method (Binkley et al. 1968).

The soluble dipeptidase hydrolyzed dipeptides, but not tripeptides (TABLE II). Its action was not restricted to dipeptides of a certain chemical composition, but it had a wide specificity (TABLE II). Assays containing a mixture of two dipeptides showed that glycyl-leucine and alanyl-serine compete for the same active sites (cf. Dixon and Webb 1964).

TABLE III

ARYLAMIDASE ACTIVITY OF THE INTESTINAL MUCOSA EXTRACT AND THE PURIFIED DIPEPTIDASE

Substrate	Assay method	$\mu\text{moles min}^{-1} \text{ ml}^{-1}$	
		Extract	Dipeptidase
Leu- β -naphthylamide	(Weber 1964)	4.4	0
Ala- β -naphthylamide	(Weber 1964)	6.1	0
Leu-p-naphthylamide	(Napel et al. 1964)	0.58	0

Some substrates besides the tripeptides (alanyl-proline, pyroglutamyl-alanine, pyroglutamyl-valine, alanyl- β -naphthylamide, leucyl- β -naphthylamide, leucyl-p-nitroanilide) were not hydrolyzed by the soluble dipeptidase (TABLE II, III, IV), showing this enzyme to be distinct from the tripeptide hydrolyzing enzymes, the intestinal prolidase (Sjöström et al. 1973), the pyrrolidonecarboxyl peptidease (Armentrout and Doolittle 1969), and the arylamidase(s) (Little and Behal 1971, Maroux et al. 1973). Neither lysyl-alanine nor tryptophyl-alanine were substrates of the enzyme although they were hydrolyzed by the lyophilized mucosal extract, which indicates that these dipeptides were hydrolyzed by other, so far undefined, (di)peptidase(s).

For some of the substrates (alanyl-tryptophan, glycyl-glutamine, glycyl-glycine, leucineamide, prolyl-alanine, tyrosyl-alanine) rather great differences in relative hydrolysis rate were found, comparing the results obtained with the lyophilized intestinal extract with that of the soluble dipeptidase, which stresses the complexity of the peptide hydrolyzing apparatus of the small intestine. For the hydrolysis of some of these substrates, distinct enzyme molecules have been suggested; namely for

glycyl-tryptophan (Smith 1948c), glycyl-glycine (Smith 1948b), prolyl-alanine (Davis and Smith 1953), and leucineamide (Smith and Spackmann 1955).

The soluble dipeptidase is strictly stereospecific, as dipeptides containing amino acids of the D-form were not hydrolyzed (TABLE IV). The necessity of free amino- and carboxyl-groups in the dipeptide is evident (TABLE IV)

TABLE IV

SPECIFICITY STUDIES ON THE PURIFIED DIPEPTIDASE

The enzyme activities are expressed as relative rate of hydrolysis of the various compounds in percentage of the hydrolysis rate of glycyl-leucine. Zero per cent activity means that the activity found was less than 0.1 per cent of that against glycyl-leucine.

Substrate	Assay method	Hydrolysis (%)
Gly-Leu	A	100
D-Ala-L-Ala	A	0
L-Ala-D-Ala	A	0
Gly-D-Leu	A	0
D-Ala-D-Ala	A	0
N-Acetyl-Leu	A	0
N-Methyl-Gly-Leu	A	0.1
pGlu-Ala	B	0
pGlu-Val	B	0
Gly-Leu-NH ₂	A	0
β -Ala-Leu	A	0
Leu- α -Ala	A	0

A, Spectrophotometric method (Josefsson and Lindberg 1965 a).

B, TNBS-method (Binkley et al. 1968).

and agrees with the findings on partially purified preparations (Smith 1948c, 1951a). The position of the amino- and carboxyl-groups in the dipeptide is also of great importance, as dipeptides containing β -alanine were not hydrolyzed by the soluble dipeptidase; a finding which agrees with earlier studies on impure enzyme preparations (Hanson and Smith 1948).

The specificity of the soluble dipeptidase from pig intestinal mucosa is similar to that found for the monkey intestinal dipeptidase (Das and Radhakrishnan 1972) and the mouse ascites tumour dipeptidase (Haymann and Patterson 1971), but quite different from the renal dipeptidase from the pig (Campbell et al. 1966).

Enzymatic properties (II).

Stability of the enzyme. The stability of the diluted dipeptidase solutions (0.2 - 20 units of dipeptidase activity per ml) used in the enzymatic studies was investigated. In 0.05 M Tris-HCl buffer (pH 8.0) and 0.1 M sodium phosphate buffer (pH 7.8) at 0 °C, the soluble dipeptidase was stable for at least 2 h. Moreover, no decrease in dipeptidase activity was observed during a 36-min period when studying the enzyme in the same Tris buffer at 25 °C. Furthermore, variations of pH in buffer between 7-9 in the presence of substrate did not influence the stability of the soluble dipeptidase.

pH-optima of the enzyme. The hydrolysis rate of glycyl-leucine and alanyl-alanine was studied in relation to changes of pH, and optima were found at pH 8.0 and 8.4, respectively. For the glycyl-leucine hydrolyzing activity in extracts of pig intestinal mucosa, a pH-optimum of 7.8 was earlier reported (Josefsson and Lindberg 1965a).

Kinetics of the enzyme. The kinetics of the soluble dipeptidase using glycyl-leucine as substrate was studied over a wide range of substrate concentrations (0.25 - 16 mM in 0.05 M Tris HCl buffer, pH 8.0, 25 °C). The rate of hydrolysis at the different substrate concentrations was measured with the method of Josefsson and Lindberg (1965a) as modified by Sjöström (1974). Within the studied substrate concentration range, the enzyme followed the kinetics of Michaelis and Menten.

In the experiments undertaken to determine the kinetic coefficients, the initial reaction velocities were determined by a continuously recording dipeptidase assay method (Sjöström 1974). Glycyl-leucine and alanyl-alanine were used as substrates, the kinetic coefficients being calculated from the experimental data with a computer (Univac 1106, Recku, Copenhagen) programmed with the statistical estimation method of Wilkinson (1961). This method presumes that the enzyme follows the kinetics of Michaelis and Menten. The K_m for glycyl-leucine (0.05 M Tris-HCl buffer,

pH 8.0, 25 °C) and alanyl-alanine (0.05 M Tris-HCl buffer, pH 8.4, 25 °C) were found to be 2.1 (S.E. 0.13) mM and 0.74 (S.E. 0.10) mM, respectively, with a satisfactory precision (Cleland 1967).

From the kinetic data and the molar extinction coefficient (III), the molecular activity of the soluble dipeptidase was calculated to be of 2.4×10^5 and 6.4×10^5 min^{-1} for glycyl-leucine and alanyl-alanine respectively, i.e. a figure similar to that reported for the purified monkey intestinal dipeptidase (Das and Radhakrishnan 1972).

Inhibitors of the enzyme. The influence of amino acids on the activity of the soluble dipeptidase was studied with glycyl-leucine as substrate. An inhibitory effect was observed for many of the amino acids tested, and it was most pronounced for histidine, leucine, isoleucine, tryptophan, and methionine; findings that agree with earlier studies on impure dipeptidase preparations (Grassmann et al. 1935, Nishi 1960, Cheeseman et al. 1970). The inhibition of leucine was further studied. The type of inhibition was found to be competitive and the K_i calculated to 5 mM, a result similar to that found for the renal dipeptidase (Campbell et al. 1967).

Glycyl-D-leucine had no inhibitory effect on the dipeptidase activity, further proving the stereospecificity of the soluble dipeptidase.

Compounds containing a free SH-group; namely, 2-mercaptoethanol, cysteine, thioglycolic acid, and cysteamine, considerably inhibited the activity of the soluble dipeptidase, as did EDTA; findings suggesting that the enzyme depends on a metal ion for its hydrolytic action.

Free sulfhydryl-groups are also of importance for the activity of the soluble dipeptidase, as inhibition was observed with sulfhydryl group reagents (Hg^{++} and p-hydroxymercuribenzoate). No inhibitory effect of DFP was noted; thus hydroxyl-groups of serine are not involved in the hydrolytic mechanism of the enzyme.

The influence of Co^{++} , Mn^{++} , Mg^{++} , and Zn^{++} on the dipeptidase activity was studied without using preincubation between enzyme and metal. With the metal concentrations used ($5\ \mu\text{M}$), a slight depression of the dipeptidase activity was observed for all the metals.

The soluble dipeptidase in its reaction with sulfhydryl-containing compounds, EDTA, and sulfhydryl-reagents, is thus very similar to the monkey intestinal dipeptidase (Das and Radhakrishnan 1972). In contrast to the findings of these investigators, no activation of the soluble dipeptidase with Zn^{++} was observed, but this difference can be explained by the different experimental procedures used.

S t r u c t u r a l p r o p e r t i e s (I I I).

Molecular weight. With gelfiltration on Sephadex G-200 (Andrews 1970) the molecular size of the soluble dipeptidase was found to correspond to a molecular weight of 104000, presuming it to have the same form and degree of hydration as the molecular weight standards used in the experiment.

In a denatured and reduced state, the soluble dipeptidase had a molecular weight of about 50000, as determined in the presence of dodecyl sulphate by polyacrylamide gel electrophoresis (Weber et al. 1972) and by gelfiltration on Sepharose 6B (Fish et al. 1970). The same result was obtained when the soluble dipeptidase was denatured but not reduced.

The molecular weight data indicate that the soluble dipeptidase is composed of two polypeptide chains of the same molecular weight associated with non-covalent bonds, but further studies on its molecular weight must be undertaken to confirm this suggestion.

Chemical composition. The amino acid composition of the enzyme was determined and is presented in Table V together with the amino acid composition of the renal dipeptidase (René and Campbell 1969) and intestinal prolidase (Sjöström and Norén 1974).

TABLE V

Amino acid composition of soluble dipeptidase from pig intestine (III), intestinal prolidase (Sjöström and Norén 1974), and renal dipeptidase (René and Campbell 1969).

Amino acid	mole %		
	soluble dipeptidase	intestinal prolidase	renal dipeptidase
Lys	3.65	3.78	6.93
His	2.56	4.03	2.40
Arg	3.76	6.34	4.13
Asx	10.11	7.92	9.39
Thr	4.23	5.10	5.30
Ser	6.06	5.87	6.87
Glu	11.03	10.02	10.30
Pro	4.84	3.78	5.04
Gly	8.87	10.58	6.06
Ala	6.89	6.64	7.72
Cys	1.79	3.96	4.17
Val	6.87	8.64	6.41
Met	1.95	2.70	1.77
Ile	5.47	3.94	3.65
Leu	9.40	7.86	9.91
Tyr	3.60	3.03	2.88
Phe	3.55	4.58	4.35
Trp	1.97	1.27	2.71

The soluble dipeptidase was investigated for its N-terminal amino acid(s) using the dansyl chloride method of Gray (1972). Proline was the only N-terminal amino acid found.

By the use of Schiff's reagent (Zacharius et al. 1969), the soluble dipeptidase was shown to contain carbohydrate (about 0.3 %) an observation that parallels the findings of two other intestinal exopeptidases (Maroux et al. 1973, Sjöström and Norén 1974).

Extinction coefficient. The $E_1^{1\%}$ value was found to be 14.0 by using amino acid analysis. The theoretical $E_1^{1\%}$ value was calculated at 14.0 from the tryptophan (molar extinction coefficient $5690 \text{ M}^{-1}\text{cm}^{-1}$) and tyrosine (molar extinction coefficient $1280 \text{ M}^{-1}\text{cm}^{-1}$) content of the enzyme. By setting the molecular weight at 104000, the molar extinction coefficient of the soluble dipeptidase was computed to $14.6 \times 10^4 \text{ M}^{-1}\text{cm}^{-1}$.

Free sulfhydryl-groups. The soluble dipeptidase was found to react readily with Ellman's reagent (Ellman 1959) in the

presence of dodecylsulphate (0.5 %, w/v). The number of sulfhydryl-groups found per dipeptidase molecule varied between 12 and 17 when the 2-mercaptoethanol was removed from the enzyme solution by gelfiltration, but when dialysis was used, lower values (8 - 11 sulfhydryl-groups per molecule dipeptidase) were obtained. As storage of the soluble dipeptidase (4 °C) without the presence of 2-mercaptoethanol reduced its sulfhydryl-group content by 40 % (unpublished), the lower values noted when removing the 2-mercaptoethanol by dialysis can be explained by the long time the soluble dipeptidase is devoid of 2-mercaptoethanol before being analysed for its sulfhydryl-group content.

The sulfhydryl-group content of the enzyme was also determined by using p-hydroxymercuribenzoate as a reagent in the presence of 0.5 % (w/v) dodecyl sulphate (Carlsen 1974), and 4.8 - 5.1 sulfhydryl-groups per dipeptidase molecule were found.

The half-cystine content of the enzyme was found to be 17 moles per mole dipeptidase, with the performic acid oxidation method (Hirs 1967). When the free sulfhydryl-group content of the soluble dipeptidase is taken into account, there are few, if any, disulfide bonds in the dipeptidase molecule; this helps to explain the lability of the enzyme and its ready reaction with Ellman's reagent (cf. pig intestinal prolidase, Sjöström and Norén 1974).

SUMMARY

The dipeptidases, enzymes of Cohnheim's erepsin, were early studied for their enzyme mechanisms. This led to the formulation of the diaffinity theory later extended to the polyaffinity theory. These enzymes were also observed to depend on metal ions for their hydrolytic activity, and a model for metal catalysis of the hydrolysis of the peptide bond was elaborated.

The physiological and medical importance of the dipeptidases has also been studied, but their role in intracellular protein catabolism and in protein digestion and absorption is still unclear, as their role in the pathogenesis of coeliac disease.

The glycyl-leucine hydrolytic activity, as other dipeptidase activities, is widely distributed among living material, the small intestinal wall being particularly rich in it.

The peptidases of the small intestine are still unknown regarding number of species, specificity, and enzymatic and structural properties, which are a prerequisite for studying their biological role.

In this thesis, a purification procedure of a soluble dipeptidase (EC 3.4.3.2) from pig small intestine is described. 10-20 mg of the purified enzyme was obtained in each preparation (30 m small intestine). It was tested to be at least 99 % pure on polyacrylamide gel electrophoresis and homogeneous in crossed immunoelectrophoresis. The specific activity of the enzyme, estimated against glycyl-leucine, varied between 1400 - 1700 units of activity per mg protein in different batches.

The soluble dipeptidase was found to be immunologically identical with a protein in a glycyl-leucine dipeptidase preparation from pig brain, which might indicate that the intestinal soluble dipeptidase is identical to the glycyl-leucine dipeptidase of the brain.

The enzyme had a wide specificity against dipeptides, but no prolidase and arylamidase activity. A low activity against leucinamide was noted. Among the dipeptides tested, alanyl-

alanine, alanyl-serine, and glycyl-leucine were hydrolyzed most rapidly. The enzyme was strictly stereospecific, required free end-groups of the dipeptides, and had no activity against dipeptides containing β -amino acids.

The enzymatic properties of the soluble dipeptidase were studied under stable assay conditions. It followed Michaelis-Menten's kinetic and showed K_m -values of 0.74 mM and 2.1 mM in its reaction with alanyl-alanine and glycyl-leucine, respectively. The pH-optima for the reaction of the two substrates were found to be 8.4 and 8.0, respectively. Several amino acids inhibited the enzyme, and kinetic analysis of the leucine inhibition showed it to be competitive in nature. The soluble dipeptidase was also inhibited by SH-compounds, EDTA, and SH-reagents, whereas DFP had no inhibitory effect on the enzyme. Glycerol and divalent metal ions only slightly influenced the activity of the enzyme.

A molecular weight of 104000 was found for the soluble dipeptidase under native conditions. Under denaturing conditions, the molecular weight was found to be about 50000. These findings suggest that the native dipeptidase might be composed of two polypeptide chains of uniform molecular weight. The two polypeptide chains might be identical, as only one N-terminal amino acid, proline, was found. The amino acid composition and the $E_{1\text{ cm}}^{1\%}$ value (14.0) of the enzyme were determined. The molar extinction coefficient was calculated to be $14.6 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$. The soluble dipeptidase was shown to contain about 0.3 % carbohydrate. The number of free SH-groups of the soluble dipeptidase was found to be about 5 when assayed with p-hydroxymercuribenzoate, but varied between 8 and 17 when Ellman's reagent was used.

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